



# Activation of c-Jun NH<sub>2</sub>-terminal kinase (JNK) signaling pathway is essential for the stimulation of hepatitis C virus (HCV) non-structural protein 3 (NS3)-mediated cell growth

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## Abstract

Hepatitis C virus (HCV) non-structural protein 3 (NS3) has been shown to affect cellular functions and is thought to contribute to the development of HCV-related hepatocarcinogenesis. In this study, we delineated part of the mechanisms whereby NS3 protein stimulates cell growth in liver (HepG2) and non-liver (HeLa) cells. The expression of NS3 protein enhanced cell growth, c-jun NH<sub>2</sub>-terminal kinase (JNK) activation, DNA binding activities of the transcription factors AP-1 and ATF-2, and c-jun expression, but not the activation of extracellular signal-regulated kinase (ERK) or p38<sup>MAPK</sup>. Whereas co-expression of NS3 with its cofactor NS4A inhibited NS3-mediated cell growth without to influence NS3-mediated JNK activation, or to affect the basal activities of ERK or p38<sup>MAPK</sup>. Pre-treatment of NS3 protein-expressing cells with JNK inhibitor, SP600125, abolished activation of AP-1 and ATF-2 and inhibited c-jun expression and induced cell growth, suggesting that JNK activation is essential for the stimulation of NS3-mediated cell growth.

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**Keywords:** HCV NS3; HCC; MAP kinase; JNK; Cell growth

## Introduction

Hepatitis C virus (HCV) still remains responsible for a significant proportion of community-acquired hepatitis. Persistent HCV infection often leads to chronic hepatitis (Alter et al., 1989; Choo et al., 1989; Tanaka et al., 1995), which is thought to be strongly associated with the development of hepatocellular carcinoma (HCC).

HCV genome has a long open reading frame, flanked with 5' and 3' untranslated region, which encodes a polyprotein precursor of about 3010–3033 amino acid (aa) residues (Takamizawa et al., 1991). This polyprotein is cleaved by both host and viral proteases to generate four structural proteins (C, E1, E2, and P7) and six non-structural

proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Neddermann et al., 1997; Takamizawa et al., 1991). Virus multiplication is dependent upon viral proteins expressed from HCV genome, including non-structural proteins that are expected to participate in genome transcription and replication (Neddermann et al., 1997).

In particular, the non-structural protein 3 (NS3) that possesses serine protease activity, which is essential for viral protein processing (Grakoui et al., 1989) and nucleotide triphosphatase–RNA helicase activity, which is essential for virus replication (Hong et al., 1996; Kim et al., 1995; Santolini et al., 1995; Suzich et al., 1993). Besides its pivotal role in viral protein processing and virus replication, the HCV NS3 protein was reported to be involved in malignant transformation of NIH3T3 cells (Sakamuro et al., 1995) as well as in the suppression of actinomycin D-induced apoptosis in NIH3T3 cells (Fujita et al., 1996). Furthermore, the role of HCV NS3 protein in the inhibition

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of PKC-mediated functions *in vitro* was demonstrated (Borowski et al., 1996). Therefore, the ability of HCV NS3 protein to affect normal cellular functions, such as cell growth, suggests a significant role for intracellular signal transduction processes in the modulation of HCV NS3 protein-induced oncogenic activity in host cells.

Changes in the level, subcellular location, and activity of kinases and phosphatases have consequences on normal cells function and maintenance (Hunter, 1994). So far, the potential role of MAP kinase signalling pathways in the modulation of the oncogenic activity of HCV NS3 protein remains largely unknown.

In the present study, we addressed the question of whether MAP kinase signalling pathways are implicated in HCV NS3 protein-mediated cell growth, and if so which MAP kinase signalling pathway is responsible for it. In this study, we demonstrated that the expression of HCV NS3 protein promotes cell growth and activates MAP kinase signalling pathway JNK. These studies suggest that JNK activation is essential for the stimulation of HCV NS3 protein-mediated cell growth in infected cells.

## Results

### *Tetracycline-regulated expression of NS3, NS3-4A complex, and NS4A in HepG2 and HeLa cells*

The expression of NS3, NS3-4A complex, and NS4A was detected by Western blotting in HepG2 and HeLa cells (HepG2-NS3, HeLa-NS3; HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A), which can be induced to express the corresponding protein under the control of tetracycline. The 70-kDa NS3 protein was detected in HepG2-NS3 and HeLa-NS3 cells (Fig. 1A) as well as in HepG2-NS3-4A and HeLa-NS3-4A cells (Fig. 1B) cultured in tetracycline-free medium. In addition to the inducible expression of NS4A in HepG2-NS4A and HeLa-NS4A cells (Fig. 1C), the NS4A was processed in HepG2-NS3-4A and HeLa-NS3-4A cells cultured in the absence of tetracycline (Fig. 1B). The expression levels of NS3, NS3-4A complex, and NS4A proteins in HepG2 and HeLa cells (HepG2-NS3, HeLa-NS3; HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A) were found to be time dependent and could be quantitatively regulated by the variation of tetracycline concentration in the culture medium (data not shown).

### *HCV NS3 protein expression contributes to the promotion of cell growth*

The growth rate of HCV NS3-transfectants was examined at 48 h intervals after the withdrawal of tetracycline from the culture medium. Then we compared the growth rate of HCV NS3 producing cells with those of HCV NS3 cells cultured in the presence of tetracycline as well as with

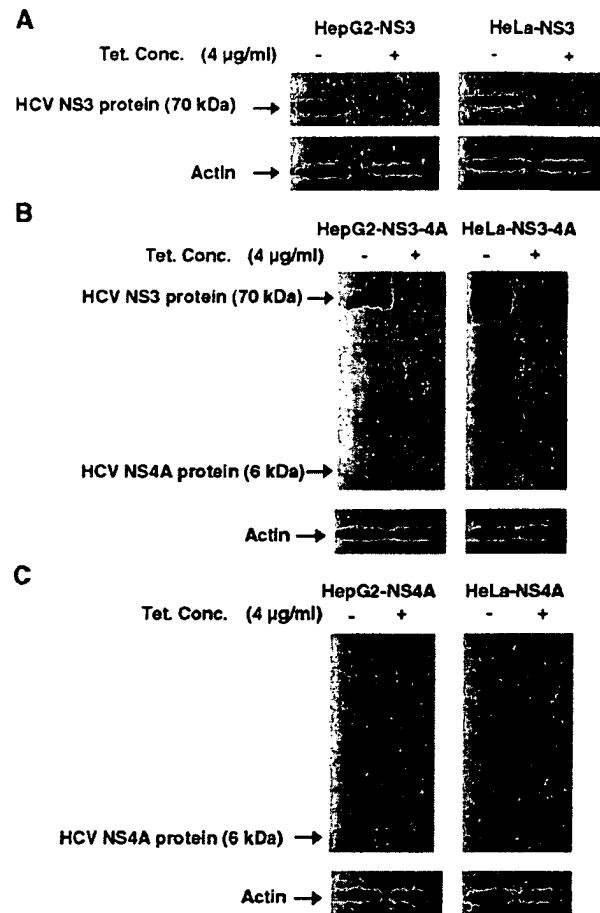


Fig. 1. Detection of NS3, NS3-4A complex, and NS4A expression in HepG2 and HeLa cells. HepG2 and HeLa cells (HepG2-NS3, HeLa-NS3, HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A) cultured in the presence (+Tc) or in the absence (–Tc) of 4 µg/ml tetracycline for 48 h. 100 µg protein was separated by 12% SDS-PAGE and analyzed by immunoblotting. (A) Expression of HCV NS3 in HepG2-NS3 and HeLa-NS3 cells. (B) Expression of HCV NS3-4A complex in HepG2-NS3-4A and HeLa-NS3-4A cells. (C) Expression of HCV NS4A in HepG2-NS4A and HeLa-NS4A cells. The same blots were reprobbed with an anti-actin antibody to compare loading and transfer.

those of HepG2 and HeLa control cells cultured in the presence or in the absence of tetracycline. The cell proliferation was measured by [<sup>3</sup>H] thymidine uptake assay (Fig. 2A) as well as by MTT assays (Fig. 2B). HCV NS3-producing cells (Figs. 2A and B) showed an enhanced growth rate against HCV NS3-transfectants cultured in the presence of tetracycline as well as against HepG2 and HeLa control cells. The enhanced growth rate of HCV NS3-producing cells was clearly significant 48 h after the withdrawal of tetracycline from the culture medium and increased thereafter up to 192 h.

To show whether the enhancement of cell proliferation by NS3 is reversible when tetracycline is readed, HepG2-NS3 and HeLa-NS3 transfectants were seeded into six-well tissue culture plates ( $2 \times 10^4$  cells/well) or into 96-well microtiter plates ( $1 \times 10^3$ ) in the presence of tetracycline.

The medium was changed at 48 h intervals up to 192 h. Subsequently, the growth rate was determined using [ $^3\text{H}$ ] uptake assay (Fig. 2C) and MTT assay (Fig. 2D) at 48 h intervals, when tetracycline was removed or readded to the medium. Based on the obtained results we could demonstrate that the enhancement of cell proliferation by NS3 is reversible when tetracycline is readded to the culture medium.

*HCV NS3 protein induces the activation of JNK, but not p38 or ERK pathways*

The ability of HCV NS3 protein to activate intracellular MAP kinase pathways was examined in both HepG2-NS3 and HeLa-NS3 transfectants, which were induced to express HCV NS3 protein for 48 h. Western blot analysis (Fig. 3)

demonstrated that the expression of HCV NS3 protein does not alter the expression of JNK, p38, or ERK either in HepG2-NS3 or in HeLa-NS3 transfectants. In contrast, in vitro kinase assay (Fig. 3) demonstrated that the expression of HCV NS3 protein either in HepG2 or in HeLa cells enhances the activation of JNK, but not those of p38 or ERK. Although the basal expression level of p38 in HeLa-NS3 cells was apparently higher than those in HepG2-NS3 cells, however, the basal level of p38 activity was more pronounced in HepG2-NS3 cells. These observations suggest that the variation at the basal expression level has no significant effect on the corresponding basal activity. Taken together, these obtained data suggest that the HCV NS3-mediated activation of JNK may be important for the stimulation of HCV NS3 protein-mediated cell growth in liver and in non-liver cell lines.

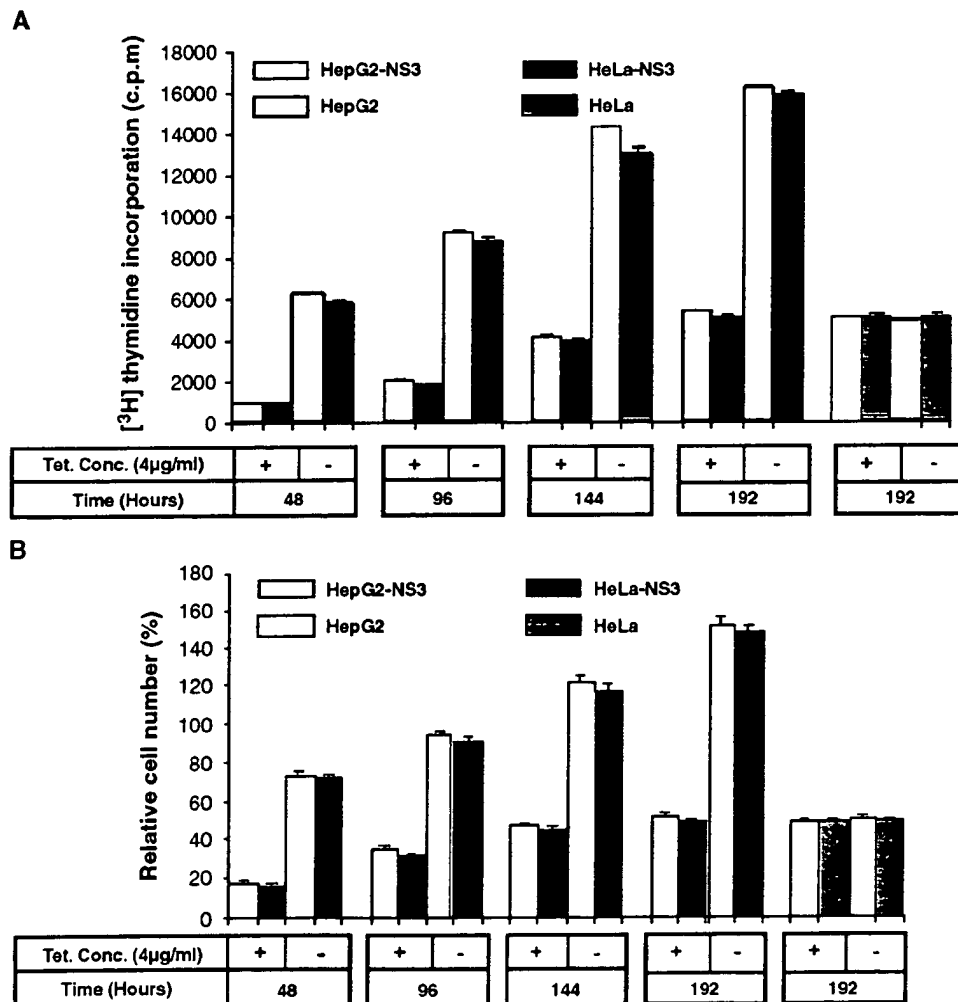


Fig. 2. Effect of HCV NS3 protein on cell growth. HepG2-NS3 and HeLa-NS3 transfectants were cultured in the presence or in the absence of tetracycline for the indicated time points. The proliferation rate was assessed by [ $^3\text{H}$ ] thymidine uptake assay (A) and MTT assay (B). The enhancement of cell proliferation by NS3 is reversible up on re-addition of tetracycline to the culture medium. The growth rate of both HepG2-NS3 and HeLa-NS3 transfectants was assessed at the indicated time points using [ $^3\text{H}$ ] thymidine uptake assay (C) or MTT assay (D). The results are means  $\pm$  SE of three independent experiments.

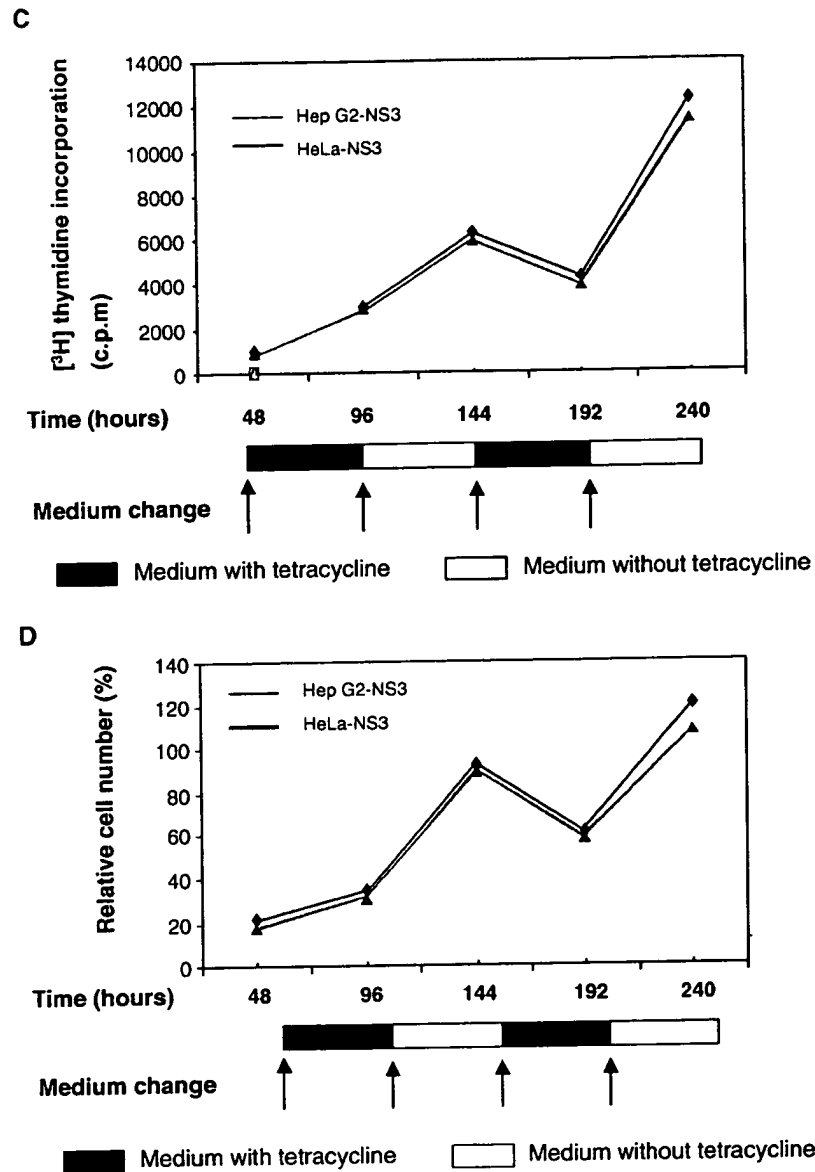


Fig. 2 (continued).

*The JNK inhibitor, SP600125, prevents activation of AP-1 and ATF-2, and the expression of c-jun, and cell growth advantage induced by HCV NS3 protein*

It has been shown that the JNK inhibitor, SP600125, specifically inhibits activation of MAP kinase JNK in response to a variety of stress stimuli (Bennett et al., 2001; Han et al., 2001). In order to determine whether the activation of JNK, by HCV NS3 protein, is directly associated with the induced cell growth of HepG2-NS3 and HeLa-NS3 transfectants, which were induced to express HCV NS3 protein, we sought to block JNK activity using SP600125 and to determine the effect on the

extent of cell growth induced by HCV NS3 protein. Results from EMSA revealed that the SP600125 inhibits HCV NS3-induced activation of AP-1 (Fig. 4A) and ATF-2 (Fig. 4B) and abolishes HCV NS3-induced expression of c-jun (Fig. 4D) in both HepG2-NS3 and HeLa-NS3 transfectants. To determine whether SP600125 affects the HCV NS3 protein-induced cell growth, the HepG2-NS3 and HeLa-NS3 transfectants as well as HepG2-Luc and HeLa-Luc transfectants were pre-treated with SP600125 1 h prior to the withdrawal of tetracycline from the culture medium, and the growth rate was determined at 48 h intervals. Results from proliferation assay (Figs. 4E and F) illustrate that although SP600125 itself does not promote

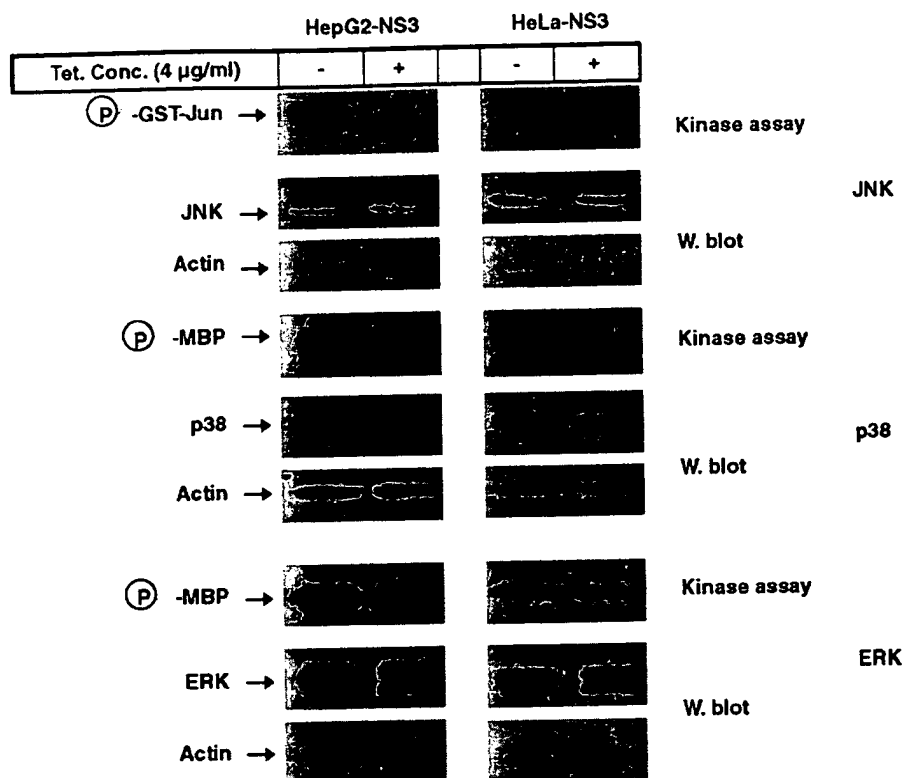


Fig. 3. Effect of HCV NS3 protein on the expression and activation of MAP kinase signalling pathways JNK, p38, and ERK. Kinase assay: equal amounts of whole cell lysates (100 µg) were immunoprecipitated using anti-JNK, p38, or ERK antibodies, respectively. Subsequently, the immune complex was subjected to an in vitro kinase assay in the presence of GST-c-jun fusion protein as a substrate for JNK or MBP as substrate for p38 or ERK. The protein kinase complex was resolved by 15% SDS-PAGE followed by autoradiography. Western blot: equal amounts of whole cell lysate were subjected to immunoblot analysis using anti-JNK, p38, or ERK antibodies, respectively. The same blots were reprobed with an anti-actin antibody to compare loading and transfer. These results are representative of three independent experiments.

proliferation of either HepG2-Luc or HeLa-Luc cells cultured in the presence or in the absence of tetracycline; however, pre-treatment with SP600125 completely abolished HCV NS3-induced proliferation in both HepG2-NS3 and HeLa-NS3 transfectants. These results suggest that the activation of the transcription factors AP-1 and ATF-2 either in HepG2-NS3 or in HeLa-NS3 transfectants, which were induced to express HCV NS3 protein, occurs as a direct consequence of HCV-induced activation of JNK. In addition, the ability of SP600125 to abrogate completely the HCV NS3 protein-mediated cell growth in HepG2 and HeLa cells suggests that the activation of JNK signalling pathway is essential for the stimulation of HCV NS3 protein-mediated oncogenic activity in liver and in non-liver cell lines.

#### *Co-expression of HCV NS3 with its cofactor NS4A in HepG2 or in HeLa cells inhibits NS3-stimulated growth advantage*

To investigate whether the cofactor NS4A influences HCV NS3-stimulated growth advantage in HepG2 and HeLa cells, stable transfectants (HepG2-NS3, HeLa-NS3;

HepG2-NS3-4A, HeLa-NS3-4A; and HepG2-NS4A, HeLa-NS4A) were cultured in the presence or in the absence of tetracycline. The cell proliferation was measured by both [<sup>3</sup>H] thymidine uptake assay (Fig. 5A) or MTT assay (Fig. 5B) at 48 h intervals up to 144 h. As shown in Figs. 5A and B, HepG2-NS3 and HeLa-NS3 cells proliferated in tetracycline-free medium more efficient than HepG2-NS3-4A and HeLa-NS3-4A cells cultured under the same conditions. Whereas, HepG2-NS3, HeLa-NS3; HepG2-NS3-4A, HeLa-NS3-4A; and HepG2-NS4A, HeLa-NS4A cells did not show any growth advantage in the presence of tetracycline (Figs. 5A and B). In contrast, HepG2-NS4A and HeLa-NS4A cells cultured in tetracycline-free medium showed growth inhibition against HepG2-NS4A and HeLa-NS4A cells cultured in the presence of tetracycline (Figs. 5A and B). This inhibition could be noted first in HepG2-NS4A and HeLa-NS4A cells cultured in tetracycline-free medium for 96 h and was more pronounced after 144 h. These results suggest that the co-expression of HCV NS4A negatively influences HCV NS3 protein-mediated growth advantage in HepG2 and HeLa cells and confirm further the inhibitory effects addressed to the expression of HCV NS4A protein in HeLa cells (Kato et al., 2002).

*Co-expression of HCV NS3 with its cofactor NS4A in HepG2 or in HeLa cells does not influence either NS3-mediated JNK activation, or the basal activity of p38 or ERK*

To determine whether the co-expression of HCV NS4A protein influences HCV NS3 protein-mediated activation or affects the basal activities of p38 or ERK, HepG2 and HeLa transfectants (HepG2-NS3, HeLa-NS3; HepG2-NS3-4A, HeLa-NS3-4A; and HepG2-NS4A; HeLa-NS4A) were cultured in the presence or in the absence of tetracycline. 48 h later, whole cell extracts were prepared and in vitro kinase assay was performed. Results from in vitro kinase assay (Fig. 6) showed that the expression of HCV NS4A protein itself does not influence the basal activity of the MAP kinases JNK, p38, or ERK either in HepG2 or in HeLa cells. In contrast, the induction of JNK activation becomes induced in both HepG2-NS3-4A and HeLa-NS3-4A cells cultured in tetracycline-free medium and was quite similar to those that noted in HepG2-NS3 or in HeLa-NS3 cells cultured under the same conditions (Fig. 6). These results indicate that the co-expression of HCV NS4A protein does not influence HCV NS3-mediated effects on JNK activation in HepG2 or in HeLa cells.

## Discussion

The results presented herein provide an insight into the possible mechanisms by which the HCV NS3 protein mediates its oncogenic activity in infected cells and support earlier findings suggesting the potential role of HCV NS3 protein in the promotion of cell growth in tumor and normal cells (Kwun et al., 2001; Sakamuro et al., 1995; Zemel et al., 2001). In this work, the HCV NS3 protein was found to induce cell growth and to activate the JNK signalling pathway, but not ERK or p38 kinases pathways in liver or in non-liver cell lines. Although the confirmation of the proliferative activity of HCV NS3 protein in liver and in non-liver cell lines, however, there are contradicting data regarding the effect of HCV NS3 protein on cell growth. Two studies demonstrated that tumor or non-tumor cells stably expressing HCV NS3 protein grow faster than their parental cell lines (Kwun et al., 2001; Zemel et al., 2001), whereas one study (Siavoshian et al., 2004) showed growth inhibition of cells transiently transfected with HCV NS3 protein. In agreement with Kwun et al. (2001), Zemel et al. (2001), we confirmed the proliferative activity of HCV NS3 protein in our cell culture model. Thus, we could demonstrate that the controlled expression of HCV NS3 protein, in liver or in non-liver cell lines, stimulates cell growth. In addition, we could show that the HCV NS3-stimulated cell growth is JNK-dependent activation.

Although the three MAP kinase signalling pathways share structural similarities, the outcome of the activation is quite different. The role of MAP kinase signalling pathways ERK, p38, and JNK in the regulation of cell proliferation is well documented in liver and in non-liver cell lines (Auer et al., 1998; Bost et al., 1999; Maher, 2001; Ogata et al., 2003; Schwabe et al., 2003; Todisco et al., 1997; Yang et al., 1999).

Since the HCV NS3 protein does not appear to affect p38 or ERK pathways, our data suggest that the HCV NS3 protein must act at a step at which the MAP kinases p38 and ERK do not converge in signal transduction pathway leading to JNK activation. Although current studies suggesting that the HCV NS3 protein may be directly involved in hepatocarcinogenesis (Ogata et al., 2003; Yang et al., 1999) by disturbing the regulation of cell proliferation, however, the mechanisms of the carcinogenesis are still puzzle. In fact, various oncogenic products are related to functional abnormalities of intracellular signal transduction pathways, which have been proved to be one of the proliferative mechanisms of cancer cells (Chang et al., 2003; Takiyara et al., 2000). In several studies, the regulation of different signal transduction processes by HCV proteins has been demonstrated (Hassan et al., 2004; Schulze zur Wiesch et al., 2003; Erhardt et al., 2002; Yang et al., 2002).

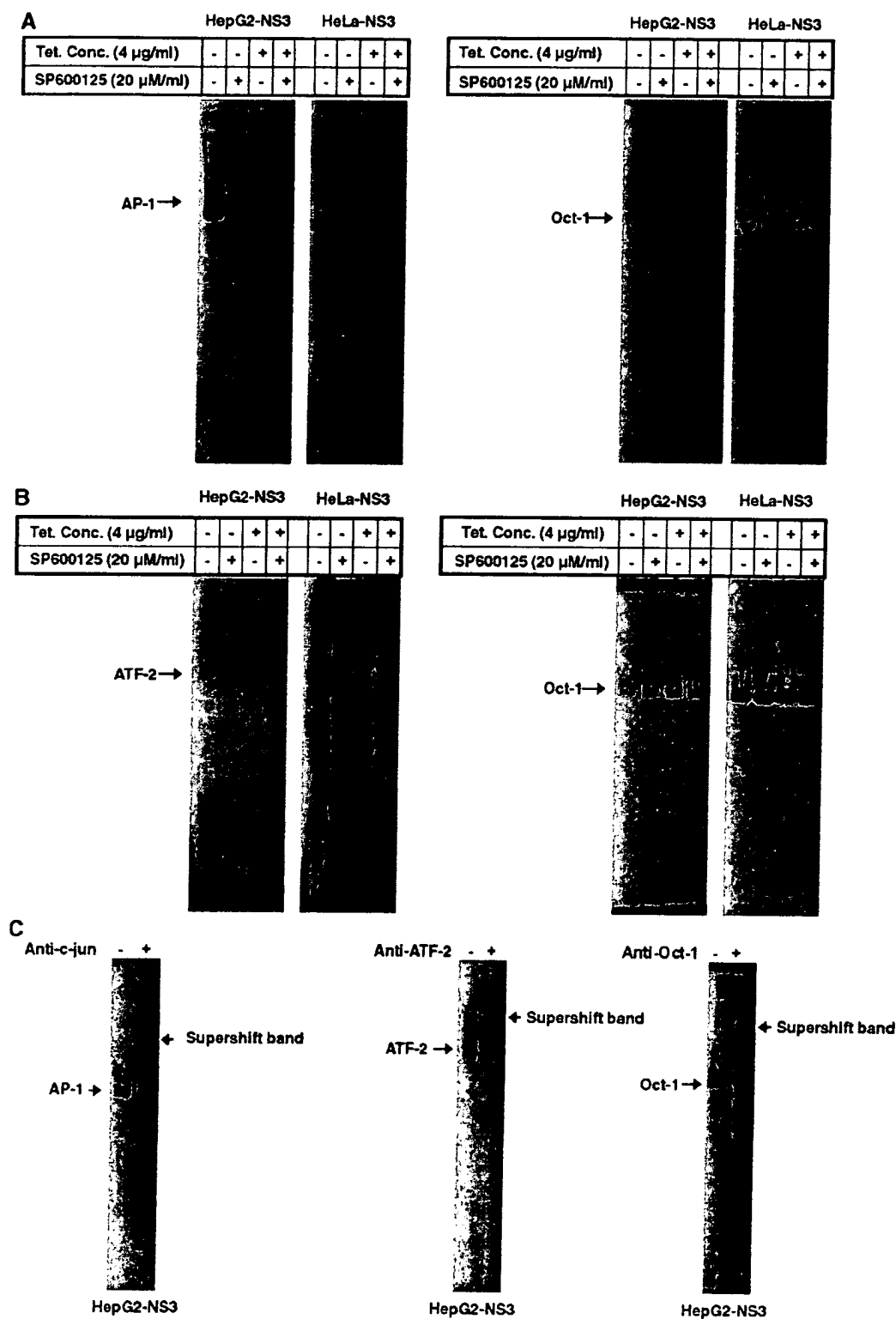
Constitutive activation of Ras/Raf/MAP kinase pathway is important for the transformation of mammalian cells (Hamad et al., 2002; Pinkas and Leder, 2002). Furthermore, the development of HCC and its progression have been shown be associated with the activation of Ras/Raf/MAP kinase pathway in both human and rodents (Ostrowski et al., 2000).

Our finding that HCV NS3 protein induces the activation of JNK signalling pathway together with the promotion of cell growth suggests a potential role for this pathway in the modulation of HCV NS3 protein-mediated oncogenic activity in host cells. Therefore, the ability of JNK inhibitor to abrogate HCV NS3-induced cell growth suggests that JNK activation is an essential component in the pathway by which HCV NS3-stimulates cell growth.

The activation of several transcription factors, such as AP-1 and ATF-2, in response to JNK activation, has been well documented (Ahmed et al., 2003; Botteron and Dobbelaere, 1998; Caelles et al., 1997; Leppa et al., 2001; Zoumpourlis et al., 2000). In many studies, the phosphorylation of c-jun by JNK has been reported to be required for the activation of AP-1, induction of c-jun expression, and modulation of cellular transformation (Angel et al., 1988; Behrens et al., 2000; Kennedy et al., 2003; Lin et al., 2003; van Dam et al., 1995). Therefore, the increase of the basal activities of AP-1 and ATF-2 as well as the basal expression of c-jun suggests that the HCV NS3 protein promotes cellular proliferation for maintenance of replication and survival (Guo et al., 1999; Lenczowski et al., 1997). In this work, we demonstrated for the first time the activation of JNK and its substrates AP-1 and ATF-2, by the expression

of HCV NS3 protein, in liver and in non-liver cell lines and delineated further the importance of the JNK signalling pathway for modulation of HCV NS3 protein-mediated cell growth. Therefore, based on our findings and in agreement with relevant studies (He et al., 2003; Kwun et al., 2001;

Sakamuro et al., 1995; Zemel et al., 2001), we suggest a potential role for the HCV NS3 protein in the development of HCV-related HCC. However, further studies with other mammalian cell lines, especially primary hepatocytes, should further clarify the importance of these results.



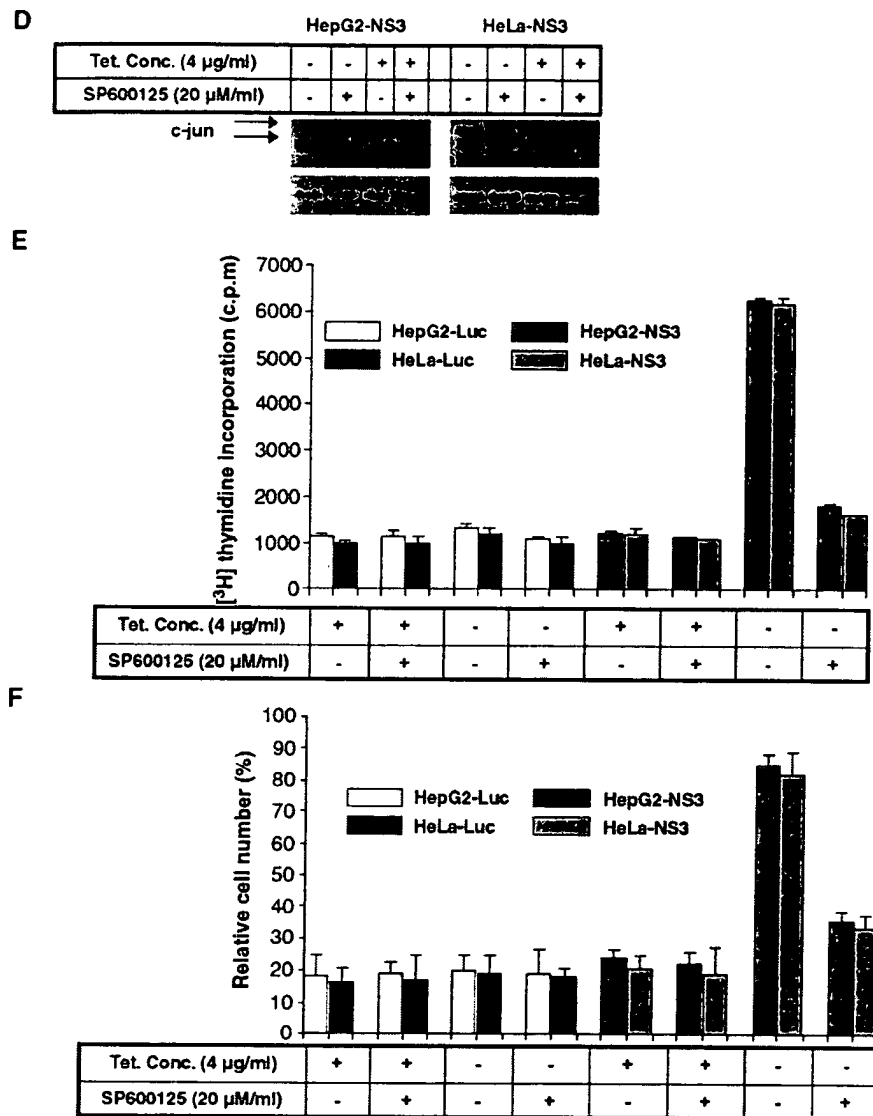


Fig. 4. Inhibition of HCV NS3 protein-induced AP-1 and ATF-2 activation, and c-jun expression, and cell growth by JNK inhibitor SP600125. HepG2-NS3 and HeLa-NS3 transfectants as well as HepG2-Luc and HeLa-Luc transfectants were pre-treated with SP600125 (20 µM/ml) 1 h prior to the cultivation in medium with (+Tc) or without (–Tc) 4 µg/ml tetracycline for the indicated time points. (A–C) The nuclear extracts were prepared and equal amounts of nuclear proteins (4 µg) were analyzed for the activation of either AP-1 (A), ATF-2 (B), and Oct-1 (A and B). The specificity of the DNA binding of AP-1, ATF-2, and Oct-1 was analyzed by both competition with unlabelled oligonucleotides (data not shown) and supershift assay (C) in HepG2-NS3 transfectants. The same analysis was performed also in HeLa-NS3 transfectants and the obtained results were comparable (data not shown). (D) The total RNAs were prepared and the expression of c-jun was analyzed by Northern blot. Membranes were then rehybridized with a GAPDH to compare loading and transfer. (E and F) Measurement of cell growth in HepG2-NS3 and HeLa-NS3 transfectants as well as in control cells HepG2-Luc and HeLa-Luc transfectants in the presence or in the absence of JNK inhibitor by either [<sup>3</sup>H] thymidine uptake assay (E) or by MTT assay (F). Results are representative of three separate experiments.

Taken together, HCV NS3-mediated JNK activity is currently understood to stimulate the transactivating potency of the transcription factors AP-1 and ATF-2 thereby increasing the expression of their target genes including c-jun (Shaulian and Karin, 2001, 2002; van Dam et al., 1995). Therefore, the HCV NS3 protein-mediated activation of AP-1 and ATF-2 may be involved in the regulation of cell-cycle progression that is reflected by a rapid increase in the proliferation rate of HepG2 and HeLa cells-expressing HCV NS3 protein.

To determine whether results obtained with HCV NS3-expressing cells are applicable to cells-expressing HCV NS3-4A complex, some of the studies were also performed with HepG2 and HeLa cells-expressing either HCV NS3-4A complex or HCV NS4A. Our data show that the co-expression of HCV NS4A results in the inhibition of HCV NS3-stimulated cell growth without to influence HCV NS3-mediated activation of JNK, or to affect the basal activities of p38 or ERK MAP kinase. Interestingly, although the expression of HCV NS4A protein itself does not influence



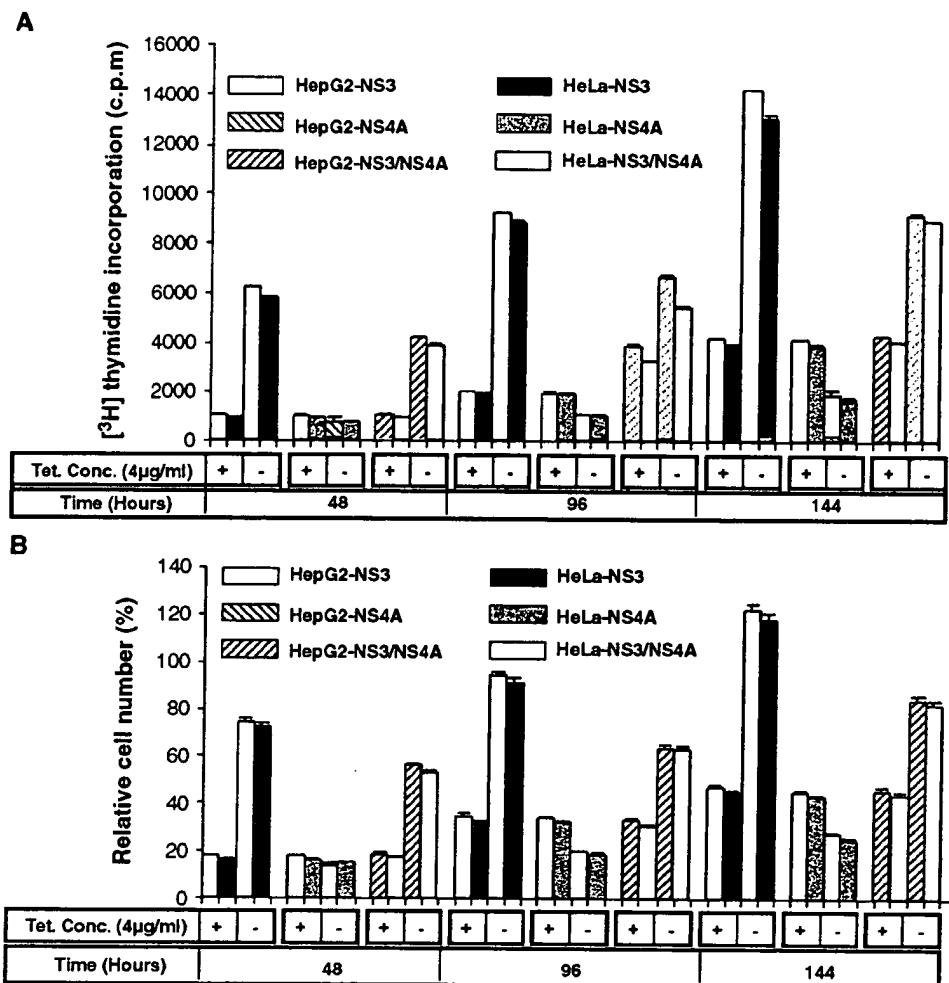


Fig. 5. Inhibition of HCV NS3-mediated cell growth by cofactor NS4A. HepG2 and HeLa cells (HepG2-NS3, HeLa-NS3, HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A) were cultured in the presence or in the absence of tetracycline for 48 h, and [ $^3$ H] thymidine uptake assay (A) and MTT assay (B) were performed. The results are means  $\pm$  SE of three independent experiments.

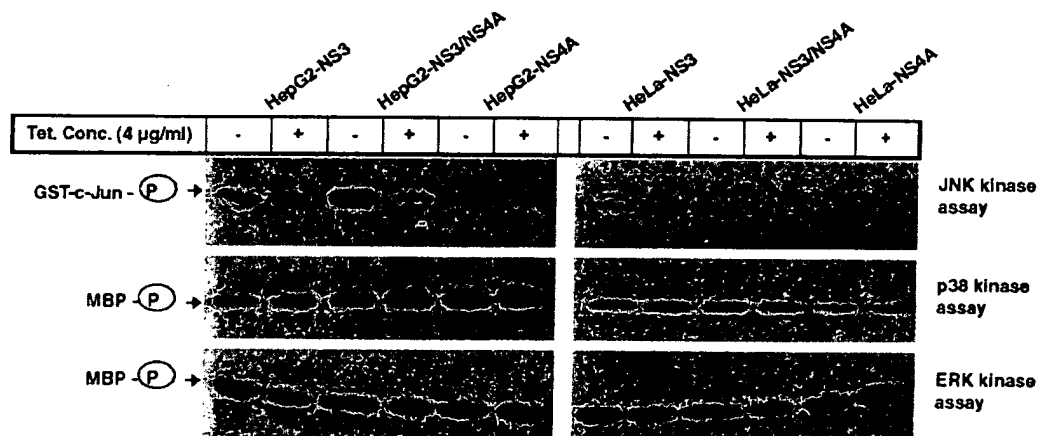


Fig. 6. Effect of NS4A co-expression on JNK, p38, and ERK MAP kinase activation. HepG2 and HeLa transfectants (HepG2-NS3, HeLa-NS3, HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A) were cultured in the presence or in the absence of tetracycline for 48 h. Cells were washed, lysed, and the JNK, p38, and ERK were immunoprecipitated from extracts using anti-JNK, p38, or ERK antibodies, respectively. JNK activity was measured by using the immune complex in a kinase assay with GST-c-jun as the substrate, whereas the activities of p38 or ERK were measured by using the immune complex in a kinase assay with MBP as substrate. These results are representative of three independent experiments.

the basal activity of the MAP kinases JNK, p38, or ERK, the expression of HCV NS4A was found to inhibit cell growth. Inhibition of cell proliferation by the expression of NS4A has been also reported (Kato et al., 2002); however, the molecular mechanisms regulating such inhibition still remain to be determined.

In summary, our data demonstrate for the first time the activation of JNK signalling pathway by HCV NS3 protein and confirm further the oncogenic activity of HCV NS3 protein in liver and in non-liver cell lines. In an attempt to further delineate part of the mechanisms whereby HCV NS3 protein mediates cell growth, we conclude that the HCV NS3 protein may be involved in hepatocarcinogenesis through the activation of JNK/AP-1 signalling pathway.

## Material and methods

### Cell lines

Human hepatoma cells (HepG2), and human cervical carcinoma cells (HeLa) (ATCC, Rockville, MD, USA). HeLa Tet-Off cells, which constitutively express the tetracycline-controlled transactivator and the RetroPack pT67 cells, were purchased from Clontech (California, USA). Cells were grown in Dulbecco's modified Eagle's medium (Sigma, Deisenhofen) supplemented with 10% fetal bovine serum.

### Extraction of RNA from sera, cDNA synthesis, and plasmid construction

Patients with high HCV RNA titers ( $10^5$ – $10^8$  copies/ml) were selected in order to obtain sufficient amount of RNA. All patients had detectable HCV RNA of the genotype 4A when we analyzed the genotyping as described (Furione et al., 1999; Hassan et al., 2004). Nucleic acids were extracted from 100  $\mu$ l of serum using QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany). The complete HCV cDNA was synthesized and cloned into the pcDNA3.1 (+) (Invitrogen, Groningen, Netherlands) as described (Chamberlain et al., 1997; Hassan et al., 2004). The following primer pairs 5'-CCA TCG ATA TGA GGG GTG GAG ACT CCT TGC-3' (HCV NS3 sense) and 5'-CCA TCG ATT GTC ACT ACC TCG AGA TCA GC-3' (HCV NS3 anti-sense); 5'-CCA TCG ATA TGA AGG GGT GGA GAC TCC TTG C-3' (HCV NS3-4A sense) and 5'-CCA TCG ATC TCC TCC ATT TCG TCC AAC TG-3' (HCV NS3-4A anti-sense); 5'-CCA TCG ATA TGG TGA CAA GTA CGT GGG TCT TG-3' (HCV NS4A sense) and 5'-CCA TCG ATC TCC TCC ATT TCG TCC AA CTG-3' (HCV NS4A anti-sense) having a start codon (bold) and a *Cla*I restriction site (underlined) were used to amplify the cDNA encoding regions for HCV NS3 (nt 3340–5244), HCV NS3-4A complex (nt 3340–5406), and HCV NS4A (nt 5244–5406) proteins, respectively. The amplified PCR products were

digested with *Cla*I and then cloned into the *Cla*I site of pRevTRE (Clontech, California, USA) to generate pRevTRE-NS3, pRevTRE-NS3-4A, and pRevTRE-NS4A plasmids.

### Generation of viruses

The packaging cell line RetroPack pT67 (CLONTECH) was grown in DMEM with 10% FCS, 2 mM L-glutamine (all from Sigma, Deisenhofen, Germany) at 37 °C, and in 5% CO<sub>2</sub>. The cells were transfected with the appropriate retroviral construct, e.g., pRev Tet-Off, pRevTRE-luciferase, pRevTRE-NS3, pRevTRE-NS3-4A, or pRevTRE-NS4A by nucleofector Kit (AMAXA BIOSYSTEMS). Forty-eight hours post-transfection, the supernatant was collected, filtered through a 0.45- $\mu$ m syringe filter, and spun at 50,000  $\times$  g for 1.5 h. Pelleted virus was resuspended in 0.1 or 0.05 the original volume of medium at 4 °C for 4 h.

### Infection of target cells

The development of HepG2-Tet-Off as well as the double stable Tet-off cell lines (HepG2-NS3, HeLa-NS3, HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, HeLa-NS4A, HepG2-Luc, and HeLa-Luc) allowing controlled expression of HCV NS3, HCV NS3-4A complex, HCV NS4A, or luciferase under the control of tetracycline was performed as described (Hassan et al., 2004). G418- and hygromycin-resistant clones, termed, HepG2-NS3, HeLa-NS3; HepG2-NS3-4A, HeLa-NS3-4A; and HepG2-NS4A, HeLa-NS4A transfectants were screened for expression of HCV NS3 protein by RT-PCR. Positive clones, with high induction efficiency, were expanded and rescreened by RT-PCR and immunoblotting using anti-HCV NS3 antibody for the expression of HCV NS3 protein or anti-HCV NS4A antibody for the expression of HCV NS4A protein, whereas luciferase transfectants were screened by luciferase assay as described (Erhardt et al., 2002; Hassan et al., 2004).

### Immunoblot

Immunoblot analysis was performed according to the standard procedures. The following antibodies were used at the indicated dilution: anti-HCV NS3 protein and anti-HCV NS4A antibodies (Research Diagnostic, Inc, USA), 1:1000; anti-JNK (Sc-474), 1:2000; anti-p38 (Sc-535), 1:2000; anti-ERK1/2 (Sc-154-G), 1:2000; and anti-actin (SC-1615), 1:5000 (Santa Cruz Biotechnology, Inc, USA).

### Northern blot

Total RNA extraction, agarose/formaldehyde gels, and Northern blot were performed as described (Erhardt et al., 2002; Hassan et al., 2004).

### Preparation of nuclear extracts

The HepG2 and HeLa cells (HepG2-NS3, HeLa-NS3, HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A) ( $2 \times 10^6$  each) were plated into a 10-cm dish (Nunc) and cultured in medium with (+Tc) or without (–Tc) 4 µg/ml tetracycline. The cells were harvested at the indicated time points and the nuclear extracts were prepared as described (Erhardt et al., 2002; Hassan et al., 2004; Lgssiar et al., 2004) with minor modification. Unless indicated otherwise, all procedures were performed at 4 °C. Briefly, cells were washed with ice-cold PBS buffer and harvested by the addition of 500 µl of buffer A (20 mM HEPES, pH 7.9; 10 mM NaCl, 0.2 mM EDTA; and 2 mM DTT) containing protease inhibitor and incubated on ice for 10 min. The supernatant was discarded after centrifugation at 14,000 rpm for 3 min. The pellet was resuspended in 50 µl of buffer C (20 mM HEPES, pH 7.9; 420 mM NaCl, 0.2 mM EDTA; 2 mM DTT; 1 mM  $\text{Na}_3\text{VO}_4$ ; and 25% glycerol) containing protease inhibitor and incubated for 20 min at 4 °C and then centrifuged at 14,000 rpm for 3 min. The supernatant was collected and stored at –80 °C until use.

### Electrophoretic mobility shift assay (EMSA)

EMSAs have been performed as described (Erhardt et al., 2002; Hassan et al., 2004; Lgssiar et al., 2004) with minor modification. Double-stranded synthetic oligonucleotides carrying binding sites for ATF-2, AP-1, or Oct-1 (SANTA CRUZ BIOTECHNOLOGY) were end-labeled with [ $\gamma$ - $^{32}\text{P}$ ] dATP (HARTMANN ANALYTIKA) in the presence of T4 polynucleotide kinase (GENECRAFT).

For binding, 4 µg nuclear extract was bound to a 0.2-ng probe in a total volume of 30 µl for 30 min at room temperature in binding buffer (10 mM Tris, pH 7.5; 50 mM NaCl, 1 mM EDTA; 1 mM  $\text{MgCl}_2$ ; 0.5 mM DTT; and 4% glycerol). The specificity of binding was analyzed by competition with an unlabeled oligonucleotide as well as with supershift assay. The competition assay was performed in the same manner, except that unlabeled probes containing either ATF-2, AP-1, or Oct-1 sequences were incubated with nuclear extracts for 20 min at room temperature before adding the labeled probes. The supershift assays were performed by incubation the nuclear extracts with antibodies to either anti-ATF-2 antibody (Sc-242), anti-c-jun antibody, a (Sc-1694), or anti-Oct-1 antibody (Sc-232) (all from SANTA CRUZ BIOTECHNOLOGY), to proof the specificities of ATF-2, AP-1, and Oct-1 DNA binding activities, respectively. The antibodies were pre-incubated for 30 min at 37 °C and then subjected to EMSA as described for ATF-2, AP-1, and Oct-1. Electrophoresis was performed for 3 h at 100 V in 0.5 X Tris-borate-EDTA running buffer at room temperature. The dried gel was visualized by exposure to high performance autoradiography film.

### In vitro kinase assays

The HepG2 and HeLa cells (HepG2-NS3, HeLa-NS3, HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A) ( $2 \times 10^6$  each) were plated into a 10-cm dish (Nunc) and cultured in medium with (+Tc) or without (–Tc) 4 µg/ml tetracycline. The cells were harvested at the indicated time points and the total cell lysates were prepared using 500 µl of buffer L (20 mM HEPES [pH 7.9], 10 mM EGTA, 40 mM  $\beta$ -glycerophosphate, 25 mM  $\text{MgCl}_2$ , 2 mM  $\text{Na}_3\text{VO}_4$ , 1 mM DTT, 1% NP-40, 5 µg apoprotinin, 1 mM leupeptin, 1 µg/ml, pepstatin, and 1 mM benzamidine). Insoluble material was removed by centrifugation, and the cell lysate was incubated with specific antibodies to anti-JNK (Sc-474), anti-p38 (Sc-535), anti-ERK1/2 (Sc-154-G), and anti-c-jun (Sc-1694) (SANTA CRUZ BIOTECHNOLOGY) for 1 h at 4 °C. The immune complexes were bound to A-sepharose (5 mg/ml in lysis buffer) by rotating overnight at 4 °C. After centrifugation, the sepharose beads were washed three times with kinase reaction buffer (80 mM HEPES [pH 7.9], 80 mM  $\text{MgCl}_2$ , 0.1 mM ATP, 2 mM  $\text{Na}_3\text{VO}_4$ , and 20 mM NaF). Kinase activity was determined by incubation with 2 µg of GST-c-Jun (Santa Cruz Biotechnology) protein as substrate for JNK or MBP (Biomol GmbH, Hamburg) protein as substrate for p38 and ERK, and 10 µCi of [ $\gamma$ - $^{32}\text{P}$ ] dATP (Hartmann Analytika) in 15 µl of kinase reaction buffer and then incubated for 30 min at 37 °C. Reactions were terminated by addition of 15 µl of sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis. The gel was dried and autoradiographed.

### $^3\text{H}$ Thymidine uptake assays

The thymidine uptake was performed as described (Erhardt et al., 2002; Hassan et al., 2004). HepG2 and HeLa cells (HepG2-NS3, HeLa-NS3, HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A) as well as HepG2-Luc and HeLa-Luc cells ( $5 \times 10^4$ /well), which can be induced to express HCV NS3, HCV NS3-4A complex, and HCV NS4A proteins and luciferase, respectively, were plated into a 6 well plates (Nunc) and cultured in medium with (+Tc) or without (–Tc) 4 µg/ml tetracycline in the presence of 0.5 µCi [ $^3\text{H}$ ] thymidine (Hartmann Analytika) and 5 µM cold thymidine. The medium was aspirated at 48-h intervals and subsequently the cells were washed twice in 5% trichloroacetic acid (TCA), followed by three washes in  $\text{H}_2\text{O}$ . The fixed cells were solubilized with 0.1 M NaOH, then mixed with 4 ml scintillant containing 0.4% TCA and counted on Packard Tricard 4000 series scintillation counter.

### MTT assay

The cell number was determined by MTT assay using cell proliferation kit (Roche, Mannheim, Germany) as

described (Erhardt et al., 2002; Hassan et al., 2004). HepG2 and HeLa cells (HepG2-NS3, HeLa-NS3, HepG2-NS3-4A, HeLa-NS3-4A, HepG2-NS4A, and HeLa-NS4A) as well as HepG2-Luc and HeLa-Luc cells ( $1 \times 10^3$ /well), which can be induced to express HCV NS3, HCV NS3-4A complex, and HCV NS4A proteins and luciferase, respectively, were plated into a microtiter plate (Nunc) and cultured in medium with (+Tc) or without (–Tc) 4 µg/ml tetracycline. The MTT assays were performed at 48 h intervals in, at least, three independent experiments in duplicate.

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